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Identification and Biological Activity of Antifungal Saponins from Shallot (*Allium cepa* L. Aggregatum Group)

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ABSTRACT: The *n*-butanol extract of shallot basal plates and roots showed antifungal activity against plant pathogenic fungi. The purified compounds from the extract were examined for antifungal activity to determine the predominant antifungal compounds in the extract. Two major antifungal compounds purified were determined to be alliospiroside A (ALA) and alliospiroside B. ALA had prominent antifungal activity against a wide range of fungi. The products of acid hydrolysis of ALA showed a reduced antifungal activity, suggesting that the compound's sugar chain is essential for its antifungal activity. Fungal cells treated with ALA showed rapid production of reactive oxygen species. The fungicidal action of ALA was partially inhibited by a superoxide scavenger, Tiron, suggesting that superoxide anion generation in the fungal cells may be related to the compound's action. Inoculation experiments showed that ALA protected strawberry plants against *Colletotrichum gloeosporioides*, indicating that ALA has the potential to control anthracnose of the plant.

KEYWORDS: *spirostanol saponin, shallot, alliospiroside, fungi, strawberry, anthracnose*

INTRODUCTION

Fungal diseases are a major limiting factor in vegetable production. These diseases have been controlled by synthetic chemical fungicides in modern agriculture. However, excessive and frequent use of fungicides has led to the development of fungicide resistance among the pathogens and to environmental pollution, which is of potential risk to animal and human health.[1] These problems highlight the need to develop alternative methods for controlling plant diseases.

Plants produce a multitude of bioactive compounds, many of which have antibiotic and antimicrobial properties.[2] The exploitation of these naturally occurring organic compounds could provide a low-cost alternative to synthetically produced fungicides and promote the local sustainable use of biological resources. This could also be useful in organic production systems, which does not allow treatment with synthetic fungicides and for which disease management techniques are highly sought after.[3]

Saponins are a major family of secondary metabolites that occur in a wide range of plant species[4] and seem to be involved in plant disease resistance.[5,6] Saponins are glycosides with a triterpenoid or steroidal aglycone. Triterpene saponins are widely distributed in nature and are typical constituents of dicotyledonous plants, whereas steroidal saponins are less distributed and usually found in many monocotyledonous families.[7] Saponins are compounds with a marked antifungal activity and are referred to as phytoanticipins because they are present in the plant tissue before a pathogen's attack.[8] The antifungal activity of saponins is due to their ability to form complexes with sterols, which results in increased membrane permeability and leakage of cell contents [5] or the induction of programmed cell death in sensitive fungal cells.[9]

The shallot (*Allium cepa* L. Aggregatum group) is an annual herbaceous plant of the Lilliaceae family and is used worldwide as a spice, food, and folk medicine. Shallot bulbs contain some steroidal saponins,[10,11] and antifungal activity of the fresh extract of shallot bulbs against medically important fungi has been reported.[12] Recently, we found that *n*-butanol extracts containing mainly saponins from bulbs of shallot have antifungal activity against a plant pathogenic fungus, *Fusarium oxysporum*.[13] These studies indicate the production of antifungal saponins by the shallot plant. Antifungal saponins (ceposide A, ceposide B, and ceposide C) were recently isolated from bulbs of the white onion, [7] which is a species very close to the shallot. However, the saponin (or saponins) corresponding to the antifungal activity of shallot remains undetermined.

The objectives of this study were to (i) evaluate the biological activity of shallot saponins on the growth of plant pathogenic fungi using an in vitro assay, (ii) identify the bioactive compounds that contribute to the antifungal activity of shallot saponins, (iii) clarify the antifungal mechanism of action of the predominant antifungal saponin, and (iv) examine the ability of the predominant antifungal saponin to inhibit plant disease.

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MATERIALS AND METHODS

Plant Materials and Fractionation. Bulbs of shallot (*A. cepa* L. Aggregatum group) were purchased at a market in Hanoi, Vietnam. Each shallot bulb was divided into the edible part (bulb scale leaf) and the basal plate. Each part was separately dried in an oven at 70 °C for a week. Shallot roots were obtained from shallot plants grown in a field at Yamaguchi University and dried in the same way. Dried samples were ground into a fine powder and defatted with hexane three times. The defatted sample was extracted with methanol three times. Combined methanol extracts were suspended into water, and the suspension was then partitioned with *n*-butanol to solubilize saponin compounds in the organic phase, leaving sugars, amino acids, and salty compounds in the water phase. The *n*-butanol extract was vacuum-dried, and the resultant brown residue was used as the crude saponin sample.

Fungal Isolates Used. Fungal strains used in this study are listed in Table 1. Fungi were cultured on potato dextrose agar (PDA).

Table 1. Fungal Isolates Used in This Study

fungus	isolate (host plant)	source
Alternaria alternata	AA1 (tomato)	our collection
Alternaria solani	AS1 (tomato)	our collection
Alternaria tenuissima	AT1 (rice)	our collection
Botrytis cinerea	BC11-1 (strawberry)	our collection
Botrytis squamosa	BS1 (Welsh onion)	our collection
Colletotrichum acutatum	CA-C1 (cherry)	our collection
C. destructivum	MAFF 305635 (*Brassica rapa* L. Perviridis group)	NIAS,[a] Tsukuba, Japan
C. gloeosporioides	CG-ST1 (strawberry)	our collection
C. graminicola	MAFF 305077 (sugar cane)	NIAS
Curvularia lunata	CL1 (rice)	our collection
Epicoccum nigrum	EN1 (rice)	our collection
Fusarium oxysporum f. sp. *batatas*	MAFF103070 (sweet potato)	NIAS
F. oxysporum f. sp. *cepae*	FOC17 (Welsh onion)	our collection
F. solani	FSOL1	our collection
F. proliferatum	MAFF 236459 (wheat)	NIAS
F. verticillioides	MAFF 239106 (banana)	NIAS
Magnaporthe oryzae	MO1 (rice)	our collection
Sclerotium cepivorum	SC1 (Welsh onion)	our collection
Thanatephorus cucumeris	TCI-2 (rice)	our collection

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Assay of Antifungal Activity. Antifungal activity was assessed with a Petri dish growth assay. PDA plates (4 cm), containing the crude or purified saponins dissolved in 80% ethanol at different concentrations, were inoculated with a 5 mm PDA plug containing the fungi. Three replica plates were used per treatment. Plates were incubated at 25 °C, and the fungal radial growth (colony diameter) was measured every 24 h. The percent of growth inhibition was calculated according to the following formula: growth inhibition (%) = (colony diameter in control plate − colony diameter in treated plate) × 100/colony diameter in treated plate.

Isolation of Antifungal Compounds. The crude saponin samples in 80% ethanol were applied to thin layer chromatography (TLC) plates (silica gel 60 F_{254}, 20 × 20 cm, layer thickness 0.25 mm, particle size of 10−20 μm; Merck, Darmstadt, Germany), developed in chloroform/methanol/water (6:3:0.5) as the mobile phase, and dried. Plates were then sprayed with anisaldehyde or Ehrich's reagent to detect steroidal saponins and furostanol saponins, respectively.[14] After development, TLC plates were examined under ultraviolet light, and spots marked on the plate were scraped off separately. Saponins were extracted from the silica gel with 80% ethanol, and the tube containing saponin solution was centrifuged at 12000*g* for 10 min. The resulting supernatant was vacuum-dried to obtain the dried saponins having a white appearance. The dried saponins were examined for purity by TLC and subjected to antifungal activity tests as described above.

Identification of the Bioactive Compound. Two spots on the TLC plate, which showed high antifungal activity, were subjected to structural analysis. The structures of the two compounds with antifungal activity were elucidated and confirmed using nuclear magnetic resonance spectroscopy (NMR). Optical rotations were taken with a JASCO DIP-1000 automatic digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded in a pyridine-d_5 solution on a JEOL ECA 500 (500, 125 MHz, respectively) spectrometer. The chemical shifts (δ) are reported in parts per million (ppm) and *J* values in hertz, using pyridine-d_5 for ^{1}H NMR (7.20 ppm) and ^{13}C NMR (123.5 ppm) as an internal standard. The HRESI-MS was recorded with a JEOL JMS-T100LP spectrometer.

Partial Acid Hydrolysis of Alliospiroside A. Alliospiroside A (1 mg) was treated at 80 °C for 6 h in a glass vial containing a solution of 1 N HCl in methanol (0.5 mL). After hydrolysis and titration to pH 7 with NaOH, the sample was vacuum-dried. The residue was dissolved in 80% ethanol, applied on TLC, and developed as described above. The antifungal activity of the HCl hydrolysates was examined as described above. The aglycon of alliospirosides A and B, ruscogenin, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Detection of Reactive Oxygen Species (ROS) in Fungal Cells. *Colletotrichum gloeosporioides* was shake-incubated in potato dextrose broth (PDB) (Becton Dickinson, Sparks, MD, USA) at 25 °C for 7 days. The culture was filtered through three layers of gauze cloth to remove mycelia. Conidial cells of the fungus were obtained by centrifugation (3000*g*, 10 min) of the supernatant. Conidial cells suspended in PDB (1×10^6 cells/mL) were shake-incubated at 25 °C for 24 h. A 500 μL aliquot of the suspension was mixed with alliospiroside A dissolved in 80% (v/v) ethanol, and the mixture was incubated for 3 h. Dead cells were detected by Evans blue staining under a light microscope. Generation of ROS in the fungal cells exposed to alliospiroside A was determined by monitoring the conversion of nonfluorescent dihydrorhodamine 123 (DHR123; Sigma-Aldrich) to fluorescent rhodamine 123 using a fluorescent microscope (BZ-9000, Keyence, Osaka, Japan).[15] Propidium iodide was also used to determine if alliospiroside A permeabilized the plasma membrane and killed the fungal cells.[16] In experiments to determine involvement of superoxide anion in the antifungal activity of alliospiroside A, conidial cell suspensions (1×10^5 cells/mL) were mixed with alliospiroside A in the presence or absence of a superoxide anion scavenger, Tiron (1,2-dihydroxy-3,5-benzenedisulfonic acid, disodium salt, monohydrate, 10 mM; Dojin, Kumamoto, Japan),[17] and incubated at 25 °C for 2 days.

Inoculation Test. For *Arabidopsis*, aliquots (100 μL) of the spore suspension of *C. destructivum* (1×10^6 spores/mL) were mixed with 1 μL of purified compound dissolved in 80% ethanol in an Eppendorf tube to give a final concentration of 10, 50, or 100 μg/mL. Spore suspension mixed with sterile distilled water was used as control. After incubation for 60 min, the spore suspensions (10 μL) were dropped onto sterile *Arabidopsis* leaves. The *Arabidopsis* leaves were subsequently kept moist in an incubator at 25 °C, illuminated in a 16:8 h light/dark cycle. Each test contained five replicates, and the tests were repeated three times.

For the strawberry (cv. Akihime), 5 mL of purified compound solutions (final concentrations of 0, 50, 100, 200, and 500 μg/mL) were sprayed onto sterile leaves of the plants. After the surface of the leaves had dried for 45 min, the plants were sprayed with 5 mL of spore suspension of *C. gloeosporioides* (1×10^6 spores/mL). The plants were sprayed with 5 mL of sterile distilled water for the control. The strawberry plants were grown in an incubator at 28 °C with 80% relative humidity and illuminated in a 16:8 h light/dark cycle for 4 days, and percent leaf area infected was estimated visually. The tests were repeated three times. The statistical analysis was carried out using IBM SPSS Statistics software version 20 (IBM Japan, Tokyo, Japan).

RESULTS

Antifungal Activity of Saponins. Yields of crude saponins extracted from the edible part (bulb scale leaf), basal plate, and root were 17.5, 22.4, and 22.1 mg/g dry weight, respectively. In a preliminary experiment, levels of antifungal activity of *n*-butanol extracts were different among the three portions of shallot bulbs: the highest antifungal activity was observed in the *n*-butanol extract from basal plates. In addition, obtaining shallot basal plates was easier than getting roots of the plant in Japan. Thus, we used the basal plates of shallot bulbs as the material for isolating antifungal compounds in the present study. *n*-Butanol extracts from the basal plates of shallots showed growth inhibition activity against all fungal isolates examined, although the extents of growth inhibition were different among the isolates: growth inhibition by the extract at 500 ppm was around 90% (*Magnaporthe grisea*), 80% (*C. gloeosporioides*), 40% (*Sclerotium cepivorum*), and 10% (*Botrytis cinerea*), respectively. No noticeable antifungal activity was shown at the concentrations of ethanol used as the solvent.

Antifungal Activity of Each Compound Separated on TLC. *n*-Butanol extracts from the basal plates and roots of shallot showed a small number of discrete spots with similar appearance on TLC after detection of saponins with *p*-anisaldehyde (Figure 1A). Two spots (1 and 2) were visualized as pink spots by Ehrich's reagent (data not shown). Ehrich's reagent is highly specific for furostanol steroidal saponins and produces pink-red spots, whereas spirostanol glycosides are not visualized.[18] Thus, the compounds corresponding to spots 1 and 2 were thought to be furostanol saponins. *n*-Butanol extracts from the edible part of shallot contained high amounts of compounds other than saponins (data not shown). Compounds relevant to spots 1−7 were recovered from the TLC plates under UV illumination and then subjected to antifungal activity test. The antifungal activity test revealed that compounds recovered from spots 3 (R_f 0.4) and 4 (R_f 0.5) had the predominant antifungal activity of shallots used in the present study (Figure 1B,C).

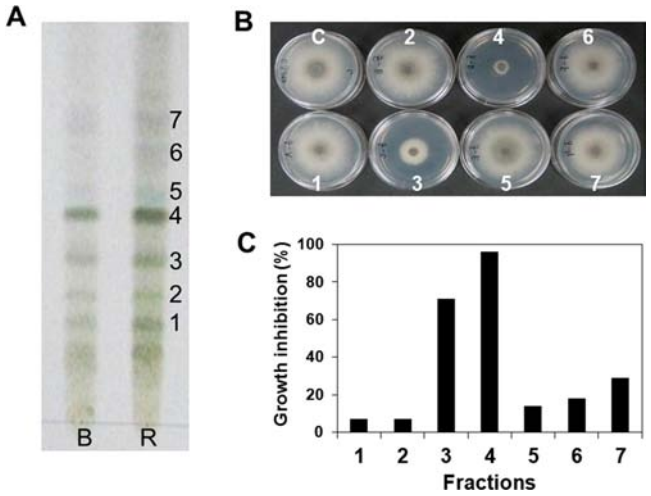

Figure 1. Antifungal activity of shallot saponins: (A) TLC profile of *n*-butanol extracts from the basal plates (B) and roots (R) stained by *p*-anisaldehyde; (B) growth of *Colletotrichum gloeosporioides* on PDA containing purified compounds (100 ppm) extracted from spots 1−7 shown in panel A; (C) percent growth inhibition of the compounds (100 ppm) relevant to spots 1−7 against *C. gloeosporioides*.

Identification of Antifungal Compounds. The compounds corresponding to spots 3 and 4 were further purified by TLC, and the resultant compounds were subjected to NMR analysis to elucidate their chemical structure. The ^{1}H NMR and ^{13}C NMR data of spots 3 and 4 were identical to those of alliospiroside B ($C_{39}H_{62}O_{13}$) [[(25*S*)-3β-hydroxyspirost-5-en-1β-yl] 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-galactopyranoside][19] and alliospiroside A ($C_{38}H_{60}O_{12}$) [[(25*S*)-3β-hydroxyspirost-5-en-1β-yl] 2-*O*-(6-deoxy-α-L-mannopyranosyl)-α-L-arabinopyranoside],[20] respectively (Figure 2).

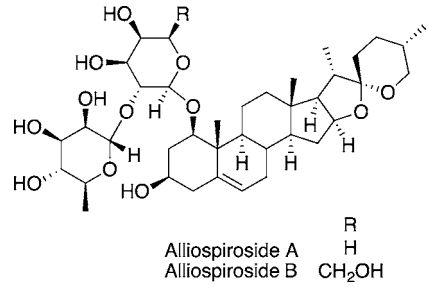

Figure 2. Structures of alliospiroside A and alliospiroside B.

Antifungal Activities of Alliospirosides A and B. Both alliospiroside A (ALA) and alliospiroside B (ALB) inhibited the growth of all plant pathogenic fungi tested in vitro. In particular, the growth of *Colletotrichum* spp. isolates, including those that were azoxystrobin-resistant,[21] was strongly inhibited (>80% growth inhibition) by ALA (data not shown). ALA showed higher antifungal activities compared to ALB against *Magnaporthe oryzae* and *Sclerotium cepivorum*, as well as *Colletotrichum* species (Figure 3). The inhibitory concentration 50% (IC_{50}) of ALA was 6 ppm against *C. gloeosporioides* and 9 ppm against *M. oryzae*. The antifungal activity of ALA was relatively low against *Botrytis cinerea*, *Fusarium oxysporum*, and *Fusarium solani*, with IC_{50} values of 100−200 ppm.

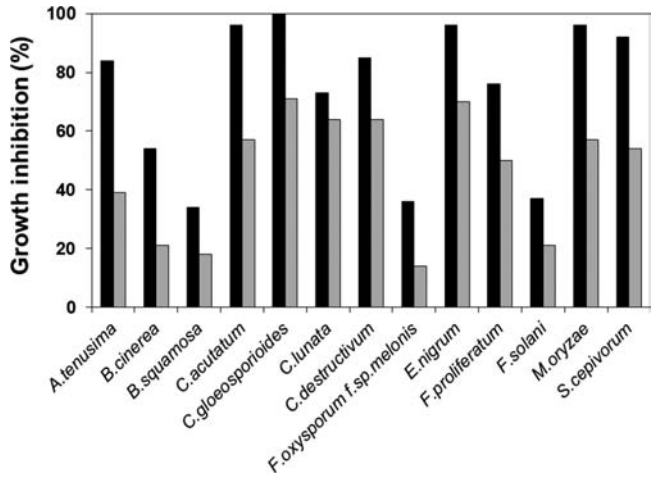

Figure 3. Antifungal activities of alliospirosides A and B. Percent growth inhibition of alliospiroside A (black bars) and alliospiroside B (gray bars) was determined on the basis of the growth on PDA containing each compound at 100 ppm.

Antifungal Mode of Action of Alliospiroside A. To determine involvement of the carbohydrate moiety of ALA in the antifungal activity, a hydrochloric acid hydrolysis product of ALA was examined for its antifungal activity. The hydrolysis

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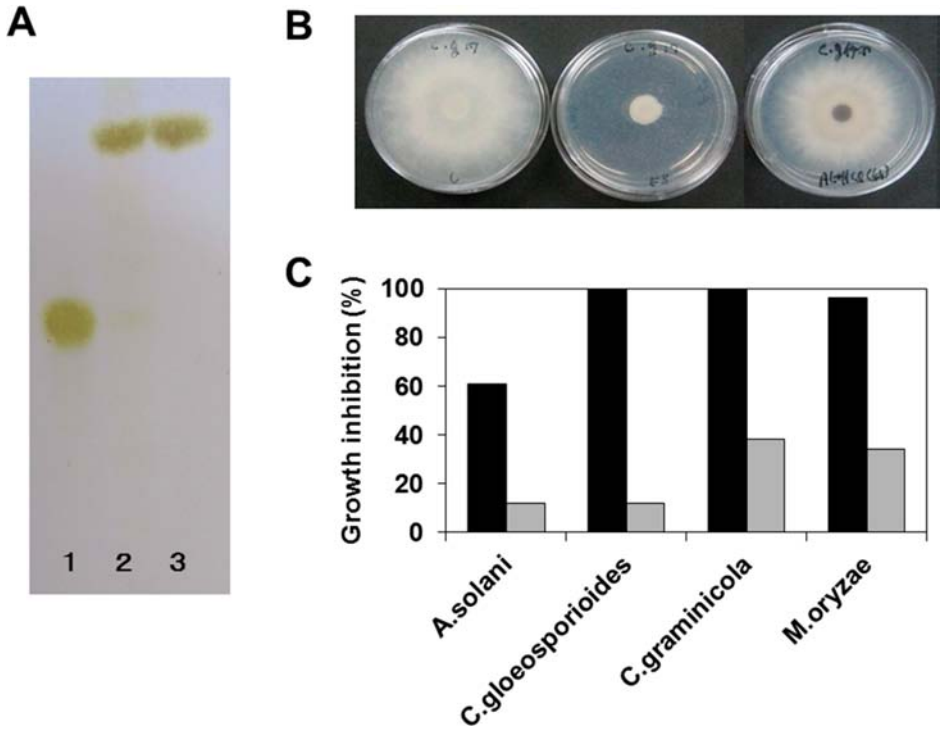

Figure 4. Antifungal activity of the hydrochloric acid hydrolysates of alliospiroside A. (A) TLC profile of the hydrochloric acid hydrolysates of alliospiroside A. Lanes: 1, alliospiroside A; 2, hydrochloric acid hydrolysates of alliospiroside A; 3, aglycon of alliospiroside A (ruscogenin). (B) Growth of *Colletotrichum gloeosporioides* on PDA containing no alliospiroside A (left), 100 ppm alliospiroside A (center), or 100 ppm hydrochloric acid hydrolysates of alliospiroside A (right). (C) Percent growth inhibition of alliospiroside A (black bars) and hydrochloric acid hydrolysates of alliospiroside A (gray bars) against plant pathogenic fungi at 100 ppm.

product (ruscogenin) showed a reduced antifungal activity (Figure 4).

ALA showed fungicidal activity in a dose-dependent manner, both to hyphae (Figure 5A) and to spores (data not shown) of the plant pathogenic fungi in PDB. To examine if ROS, which have been known to be involved in the fungicidal action of saponin,[9] are involved with the antifungal activity of ALA, *C. gloeosporioides* cells treated with ALA were stained with DHR 123. Fungal cells treated with 500 ppm ALA, at which most fungal cells were killed by the compound, showed a rapid production of ROS (Figure 5B). Staining of fungal cells with propidium iodide, which stains fungal cells with damaged membranes, also showed a similar staining pattern (data not shown). The extent of dead cells stained with Evans blue dye (Figure 5A) seemed to correlate with the level of ROS production (Figure 5B). The antifungal action of ALA was partially inhibited by Tiron, a superoxide scavenger (Figure 5C).

Inhibition of Infection of Anthracnose Pathogens by Alliospiroside A. ALA protected strawberry plants from attack by *C. gloeosporioides* sprayed on the plants at concentrations >100 ppm before inoculation of the pathogen (Figure 6). *A. thaliana* plants inoculated with *Colletotrichum destructivum* spores showed reduced symptoms by <50% when the spores were pretreated with ALA at 50 ppm (data not shown).

DISCUSSION

No spirostanol saponin, including either ALA or ALB, has been isolated from the shallot until now, although furostanol saponins have been found in the bulbs of the shallot.[11] We revealed in the present study that spirostanol saponins, ALA and ALB, are contained in the basal plate and root of shallot and that they are predominant antifungal saponins against plant pathogenic fungi. ALA and ALB are mainly contained in the basal plate and root of the shallot, where few researchers have focused in previous studies,[7,12] which may explain why there has been a lack of information about them. Indeed, the edible part (bulb scale leaf) of the shallot, generally used as the material for shallot studies,[7,10−12] contains very small amounts of ALA and ALB, as described in the present study.

Lanzotti et al.[7] isolated antifungal furostanol saponins (ceposides A−C) from the bulb of the white onion, aspecies very close to shallot. Although furostanol saponins are also contained in the basal plate and root of the shallot (spots 1 and 2 shown in Figure 1), their antifungal activity was very low in the present study (Figure 1C). This suggests that the furostanol saponins contained in the basal plate of shallots may be different from those contained in white onions reported by the authors.

ALA is present in some tissues of plants other than *Allium* species: the fresh stems of *Dracaena concinna*[22] and *Dracaena angustifolia*[23] and the bulbs of *Ornitogalum thyrsoides*.[24] Xu et al.[23] reported that ALA from *D. angustifolia* exhibited selective antifungal activity against an animal pathogen, *Cryptococcus neoformans*, with IC_{50} of 20 ppm. However, the antifungal activity of ALA against plant pathogenic fungi has not been documented. We showed that ALA and ALB possess antifungal activity against a wide range of plant pathogenic fungi, among which anthracnose pathogens *Colletotrichum* spp. were highly sensitive to ALA. It is interesting that ALA strongly inhibited azoxystorbin-resistant *C. gloeosporioides* in vitro because azoxystrobin-resistant *C. gloeosporioides* has become a growing problem for strawberry production in Japan.[21] Thus, we examined whether ALA can inhibit the incidence of

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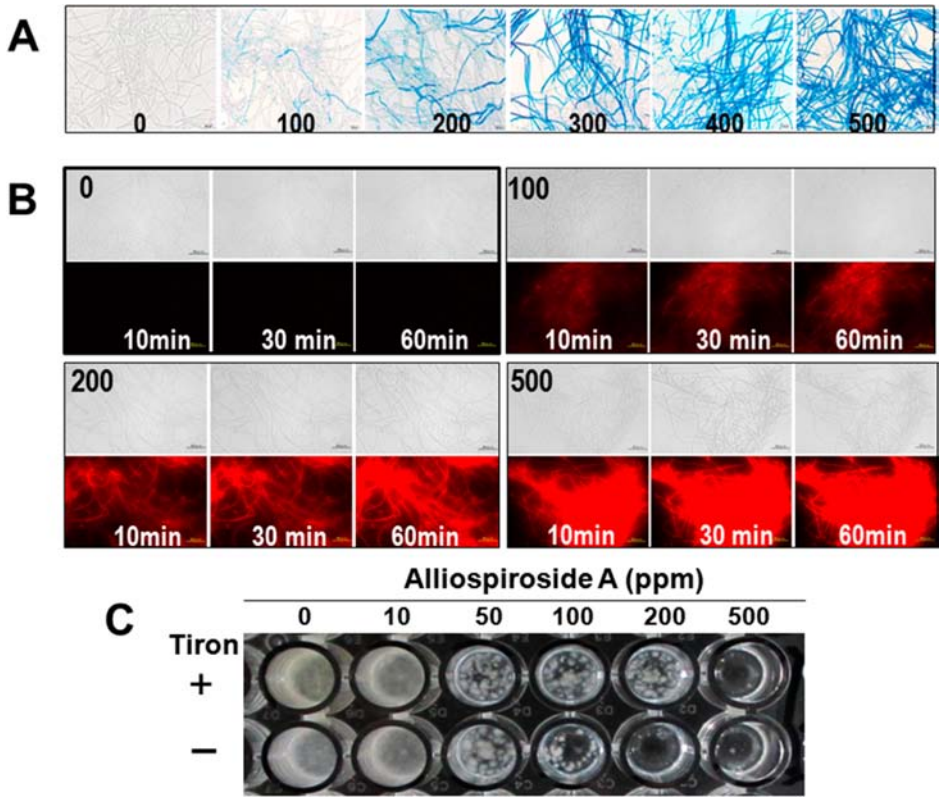

Figure 5. Fungicidal action of alliospiroside A at different concentrations: (A) Evans blue staining of *Colletotrichum gloeosporioides* exposed to different concentrations (0, 100, 200, 300, 400, and 500 ppm) of alliospiroside A; (B) phase contrast (upper panels) and epifluorescence microscopy (lower panels) microscopy of *C. gloeosporioides* cells exposed to different concentrations (0, 100, 200, and 500 ppm) of alliospiroside A for different time periods (10, 20, and 60 min); (C) inhibition of fungicidal action of alliospiroside A against *C. gloeosporioides* cells by a superoxide scavenger, Tiron (10 mM).

anthracnose of strawberry. ALA significantly inhibited the disease when it was presprayed onto the plants at a concentration >100 ppm before inoculation of azoxystrobin-resistant *C. gloeosporioides*. The result suggests that ALA has the potential to inhibit the disease caused by the pathogen. ALA also suppressed anthracnose of *A. thaliana* caused by *C. destructivum*. These results suggest that ALA would be a useful compound to suppress plant diseases caused by *Colletotrichum* spp.

The biological activity of saponins is more related to their carbohydrate moiety, although the involved mechanisms have not yet been characterized.[25] Our data also support this notion, as the hydrolysis product of ALA (ruscogenin) showed dramatically reduced antifungal activity. It is difficult to interpret the role of the carbohydrate moiety in the antifungal activity of saponins in the present study. However, our data suggest that only one sugar residue of the carbohydrate moiety, L-arabinose in ALA and L-galactose in ALB, is concerned with different levels of antifungal activity between ALA and ALB. These two compounds may be useful for studying the relationship between chemical structures and biological activities of saponins with a single sugar chain (monodesmosides) at C-1 of ruscogenin.

ALA and ALB inhibited the growth of soilborne pathogens, such as *Fusarium proliferatum* and *S. cepivorum*. Antifungal compounds other than ALA and ALB were also found in basal plates and roots of the shallot, as shown in the present study (Figure 1). These compounds may function along with each other as a chemical barrier against such soilborne pathogens invading the shallot through the root and basal plate. ALA and ALB are also contained in the collective fruit (ripened seeds) in the onion.[19,20] Thus, ALA and ALB likely are important in the defense system of collective fruit, preventing fungal attack of flowers and seeds, as well as the underground part of plants belonging to *A. cepa* L.

ROS production occurs in fungal cells treated with α-tomatine, a spirostanol saponin of tomato.[9] We found, in the present study, that ROS was also produced in *C. gloeosporioides* cells treated with ALA. Interestingly, ROS production levels coincided with the extent of cell death, suggesting involvement of ROS production in the fungicidal action of ALA. In addition, the fungicidal action of ALA was partially inhibited by a superoxide anion scavenger, Tiron. The major endogenous source of ROS, including the superoxide anion, is mitochondria, in particular, damaged mitochondria.[26] Loss of membrane integrity in the fungal cells exposed to ALA was suggested by the increased uptake of the fluorescent dye, propidium iodide. These results suggest that ALA causes damage to the mitochondrial membrane, leading to celluar dysfunction and eventually cell death.

Plant-derived compounds, such as ALA and ALB, have the advantage of limited negative impacts on human health and the environment. However, further studies are required to determine their toxicity against animals before application of the compounds in the field, as some saponins have been reported to be toxic to animals.[27]

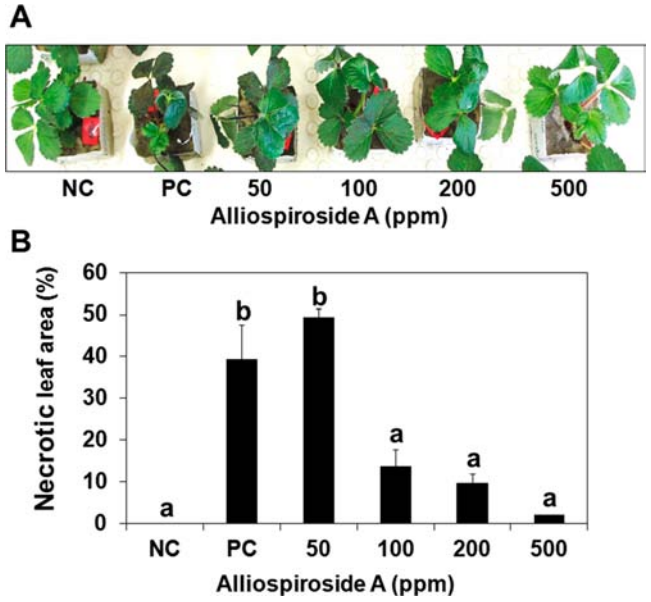

Figure 6. Inhibition of infection of strawberry with azoxystrobin-resistant *Colletotrichum gloeosporioides* by alliospiroside A. (A) Anthracnose symptoms of strawberry pretreated with different concentrations (50, 100, 200, and 500 ppm) of alliospiroside A. NC, negative control; PC, no-treatment control. (B) Necrotic leaf area (%) assessed 4 days after inoculation by visually estimating the percent of leaf showing necrosis. Values represent the means of three replicates, with six leaves per replication in each treatment. Error bars are standard error of the mean. Columns with same letters are not significantly different according to Tukey's honestly significant difference (P = 0.05).

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Notes

The authors declare no competing financial interest.

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